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(54) Title: TELOMERASE INHIBITORS AND METHODS OF THEIR USE

(57) Abstract: Substituted flavone compounds, compositions, and methods of inhibiting telomerase activity and treatment of telomerase mediated conditions or diseases are provided. The methods, compounds and compositions of the invention may be employed alone, or in combination with other pharmacologically active agents in the treatment of conditions or diseases mediated by telomerase activity, such as in the treatment of cancer.

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TELOMERASE INHIBITORS AND METHODS OF THEIR USE

Cross-reference to Related Applications

The application claims priority from U.S. Application No. 60/200,1171, filed April 27, 2000, incorporated herein by reference in its entirety.

Field of the Invention

The present invention relates to substituted flavone and isoflavone compounds that inhibit telomerase activity, to pharmaceutical compositions containing the compounds and to the use of the compounds and compositions, alone or in combination with other pharmaceutically active agents, in the treatment of telomerase-mediated conditions or diseases, such as cancer.

Background of the Invention

Telomerase catalyzes the synthesis of telomeres. Telomeres are characteristic tandem repeats (TTAGGG in humans) found at the ends of most eukaryotic chromosomes, that may be 15-25 kilobases long in human germline cells. With each cell division, about 60-100 bases are lost from the ends of the chromosomes, and as the telomeres shorten toward non-existence, cells eventually reach crisis and apoptosis is triggered (Harley *et al.*, (1991) Mutation Res. 256: 271-282).

Telomerase is a ribonucleoprotein reverse transcriptase that contains its own RNA template for the synthesis of telomeric DNA (Blackburn (1992) Annu. Rev. Biochem., 61:113-129). Telomerase acts to replace telomeric repeats that are lost during cell division. However, while telomerase is absent in normal somatic cells, the enzyme is present in stem and germline cells of normal tissues, and in over 85% of tumors (Kim *et al.*, (1994) Science, 266:2011-2014). Thus, drugs targeted towards telomerase potentially will have a high selectivity for tumor over healthy tissues. Consequently, inhibition of the telomerase enzyme has been proposed as a new approach to cancer therapy.

The inhibition of telomerase activity by antisense strategies directed towards the telomerase RNA component, for example peptide nucleic acids (see U.S. Patent No.: 6,046,307) and phosphorothioate oligonucleotides has been reported. Since telomerase is a reverse transcriptase, the use of inhibitors of reverse transcriptases, such as AZT, and other nucleosides has also been reported. Telomerase inhibition by cisplatin, possibly due to crosslinking of the telomeric repeat sequences, is also known (Burger et al., (1997) Eur. J. Cancer 33: 638-644).

The flavones and isoflavones comprise a group of structurally related compounds many of which occur naturally. In nature, flavones are usually in glycosylated form, and are commonly referred to as flavonoids. The flavonoids are widely distributed among plants, including most all citrus fruits, rose hips and black currants. Such compounds are generally isolated from the rinds of oranges, tangerines, lemons, limes, kumquats and grapefruits by commercial extraction methods. Certain flavonoids have been determined to be involved with homeostasis of the walls of small blood vessels. In addition, these compounds have been found to contribute to the maintenance of normal blood vessel conditions by decreasing capillary permeability and fragility. Certain flavonoids have also been found to have activity as a histamine release blocker (treatment of allergies), a xanthine oxidase inhibitor (treatment of gout), an aldose reductase inhibitor (prevention of diabetic complications), a phospholiphase A2 and lipoxygenase inhibitor (anti-inflammatory), an aerobic glycosis inhibitor (an anti-cancer agent), and a tumor necrosis factor potentiator (an antiviral agent). Recently, some flavonoids have been shown to have anticancer activity (*Cancer Research* **48**: 5754, 1988) and chemopreventive activity in some tumors (*J. Nat. Prod.* **53**: 23, 1990). In particular quercetin, a flavonoid almost ubiquitous in plants, has shown some inhibiting activity on the proliferation of human leukemia cells (*Br. J. of Haematology* **75**: 489, 1990) and on other cell lines (*Br. J. Cancer* **62**: 942, 1990; *Int. J. Cancer* **46**: 1112, 1990; *Cancer Chemother. Pharmacol.* **28**: 255, 1991; *Gynecologic Oncology* 13: 1991 (1992)) besides a synergistic activity with the conventional chemotherapeutics. Although the mechanism of such an inhibiting action on

proliferation is unknown, it has been suggested to be related to the interaction of this flavonoid with the estrogen receptors of type II (*J. Steroid Biochem.* **30**: 71, 1988). It has been reported that the flavonoid apigenin (5,7,4'-trihydroxyflavone) inhibits telomerase activity (*J. Biochem. And Mol. Biol.* 31(4):339, 1998). The differential inhibitory effect of various flavone, flavanone and anthocyanidin compounds on reverse transcriptases from Rauscher murine leukemia virus (RLV) and human immunodeficiency virus (HIV) is disclosed in Ono et al., "Differential Inhibitory Effects of Various Flavonoids on the Activities of Reverse Transcriptase and Cellular DNA and RNA Polymerases" *Eur. J. Biochem.* **190**:469-476 (1990). In addition, Chu et al., "Inhibitory Effects of Flavonoids on Moloney Murine Leukemia Virus Reverse Transcriptase Activity" *J. Nat. Products* **55(2)**:179-183 (1992) discloses that various flavonoid compounds exhibit inhibitory activity against Moloney murine leukemia virus (MMLV) reverse Transcriptase.

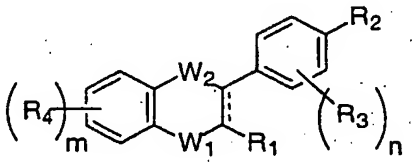
The identification of compounds that inhibit telomerase activity provides important benefits to efforts at treating human disease. Unfortunately, few such compounds, especially compounds that have high potency or activity and can be administered orally, have been identified and characterized. Hence, there remains a need for compounds that act as telomerase inhibitors that have relatively high potency or activity and are orally bioavailable, and for compositions and methods for treating cancer and other telomerase-mediated diseases. The present invention meets these and other needs.

Summary of the Invention

The present invention provides methods, compounds and compositions that are unique, specific, and effective for treating telomerase-mediated disorders, such as malignant conditions by targeting cells having telomerase activity. The methods, compounds, and compositions of the invention can be applied to a wide variety of malignant cell types and avoid the problems inherent in current cancer treatment modalities which are non-specific and excessively toxic.

In a first aspect, the present invention is based on the surprising finding that certain known 3-substituted flavone compounds and 2-substituted isoflavone compounds, as well as the new flavone and isoflavone derivatives disclosed herein, are effective in the inhibition of telomerase activity in cells. A related aspect of the present invention is the discovery that 3-substituted flavone compounds and 2-substituted isoflavone compounds are selective and effective in the treatment of conditions or diseases mediated by telomerase activity *in vivo*, such as, for example, in the treatment of cancerous cells, while not affecting the viability or life cycle of normal cells. Thus, another aspect of the present invention provides methods of inhibiting telomerase activity in a patient suffering from a telomerase-mediated condition or disease, comprising administering to the patient a therapeutically effective amount of a telomerase inhibiting substituted flavone compound, or a pharmaceutically acceptable salt thereof.

In another aspect, the present invention provides methods, compounds and compositions for treating a telomerase-mediated condition or disease, such as cancer, in which a patient is administered a therapeutically effective amount of a compound having the following formula (I):



(I)

wherein one of W_1 and W_2 is C=O and the other of W_1 and W_2 is O or N;

⁘ is a single or double bond;

R_1 is hydroxyl, carboxyl or sulfo;

R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen, halo, alkyl, haloalkyl, hydroxyl, alkoxyl, cyano, nitro, amino, alkylamino, dialkylamino, carboxyl, and alkoxycarbonyl;

m and n are independently 0, 1, 2, 3 or 4;

and the pharmaceutically acceptable salts thereof.

The new compounds of the invention have many valuable uses as inhibitors of deleterious telomerase activity, such as, for example, in the treatment of cancer in humans. The pharmaceutical compositions of this invention can be employed in treatment regimens in which cancer cells are killed, *in vivo*, or can be used to kill cancer cells *in vitro* or *ex vivo*. Thus, this invention provides therapeutic compounds and compositions for treating cancer, and methods for inhibiting telomerase activity in cells and for treating cancer and other telomerase-mediated conditions or diseases in humans and other mammals (e.g., cows, horses, sheep, steer, pigs and animals of veterinary interest such as cats and dogs).

Brief Description of the Drawings

FIGURE 1 depicts a synthetic route for the preparation of flavone compounds of the invention; and

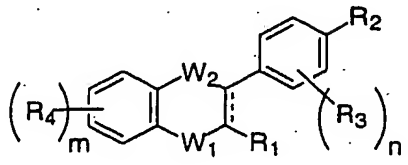
FIGURE 2 depicts a synthetic route for the preparation of isoflavone compounds of the invention.

Detailed Description of the Preferred Embodiment

As set forth above, the present invention provides in one aspect methods of inhibiting telomerase activity in cells. In other aspects, the present invention provides methods for inhibiting telomerase activity in a patient suffering from a telomerase-mediated condition or disease, comprising administering to the patient a therapeutically effective amount of a telomerase inhibiting hydroxyflavone derivative compound, or a pharmaceutically acceptable salt thereof.

In other aspects, the present invention provides methods, compounds and compositions for treating a telomerase-mediated condition or disease, such as cancer, in which a patient is

administered a therapeutically effective amount of a compound having the following formula (I):



(I)

wherein one of W_1 and W_2 is C=O and the other of W_1 and W_2 is O or N;

is a single or double bond;

R_1 is hydroxyl, carboxyl or sulfo;

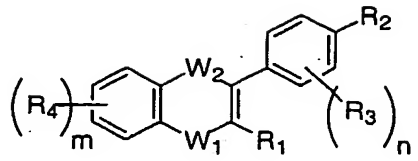
R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen, halo, alkyl, haloalkyl, hydroxyl, alkoxy, cyano, nitro, amino, alkylamino, dialkylamino, carboxyl, and alkoxycarbonyl;

m and n are independently 0, 1, 2, 3 or 4;

and the pharmaceutically acceptable salts thereof.

The compounds of structure I are referred to herein as "3-substituted flavone" compounds, in reference to the R_1 substituent at position 3 of the flavone nucleus (see numbering scheme set forth in structure VI, below), or as "2-substitued isoflavone" compounds in reference to the R_1 substituent at position 2 of the isoflavone nucleus (see structure V, below).

In other aspects, the present invention provides methods, compounds and compositions for treating a telomerase-mediated condition or disease, such as cancer, in which a patient is administered a therapeutically effective amount of a compound having the following formula (II):



(II)

wherein one of W_1 and W_2 is C=O and the other of W_1 and W_2 is O or N;

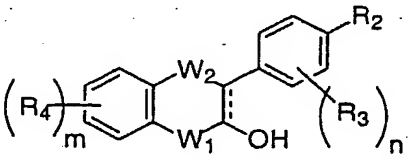
R_1 is hydroxyl, carboxyl or sulfo;

R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen, halo, alkyl, haloalkyl, hydroxyl, alkoxy, cyano, nitro, amino, alkylamino, dialkylamino, carboxyl, and alkoxycarbonyl;

m and n are independently 0, 1, 2, 3 or 4;

and the pharmaceutically acceptable salts thereof.

In yet other aspects, the present invention provides methods, compounds and compositions for treating a telomerase-mediated condition or disease, such as cancer, in which a patient is administered a therapeutically effective amount of a compound having the following formula (III):



(III)

wherein one of W_1 and W_2 is C=O and the other of W_1 and W_2 is O or N;

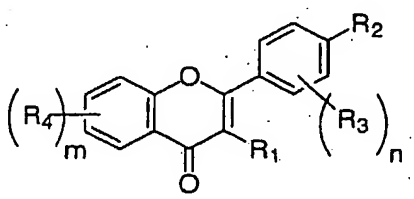
⋰ is a single or double bond;

R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen, halo, alkyl, haloalkyl, hydroxyl, alkoxy, cyano, nitro, amino, alkylamino, dialkylamino, carboxyl, and alkoxycarbonyl;

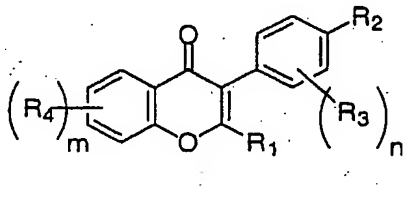
m and n are independently 0, 1, 2, 3 or 4;

and the pharmaceutically acceptable salts thereof.

In other embodiments, the new substituted flavone compounds of the present invention have the general structure shown as formula IV below, and the new substituted isoflavone compounds of the present invention have the general structure shown as formula V below:



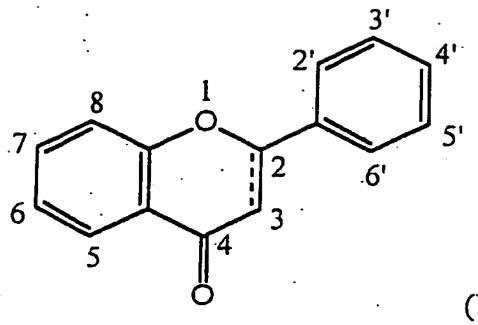
(IV)



(V)

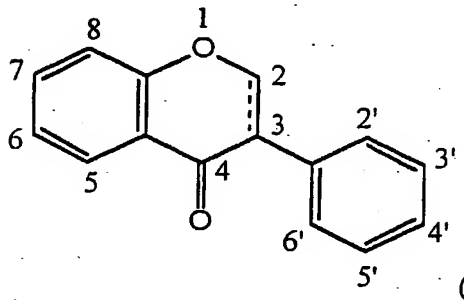
and their pharmaceutically acceptable salts, wherein R_1, R_2, R_3 and n have the meanings defined above.

As used herein, the term "substituted flavone" compounds or derivatives useful in the practice of the invention is intended to include both flavone derivatives and isoflavone derivatives. The term "flavone derivative" as used herein refers to substituted compounds of the general formula VI:



(VI)

The term "isoflavone derivative" as used herein refers to substituted compounds of the general formula VII:



(VII)

As shown in structures VI and VII, when the pendant phenyl ring is attached at the 2-position, the derivatives are flavone derivatives. When the pendant phenyl ring is attached at the 3-position, the derivatives are isoflavone derivatives.

Many flavones are naturally-occurring compounds, but synthetic flavones and isoflavones are also encompassed by the present invention. Naturally-occurring compounds useful in the practice of the invention include compounds of structure VI having hydroxyl groups in the positions shown in Table 1:

Table 1

Naturally Occurring Flavones

OH substitution positions

	3	5	7	8	2'	3'	4'	5'
Flavone	-	-	-	-	-	-	-	-
Flavonol	+	-	-	-	-	-	-	-
Chrysin	-	+	+	-	-	-	-	-
Galangin	+	+	+	-	-	-	-	-
Apigenin	-	+	+	-	-	-	+	-
Fisetin	+	-	+	-	-	+	+	-
Luteolin	-	+	+	-	-	+	+	-
Kaempferol	+	+	+	-	-	-	+	-
Quercetin	+	+	+	-	-	+	+	-
Morin	+	+	+	-	+	-	+	-
Robinetin	+	-	+	-	-	+	+	+
Gossypetin	+	+	+	+	-	+	+	-
Myricetin	+	+	+	-	-	+	+	+

Flavone or isoflavone derivatives of the invention may be modified to comprise any of a variety of functional groups, such as hydroxyl, alkyl, alkoxyl, amino and/or halo groups. Preferred flavones comprise a C_{2-3} double bond and one or more hydroxyl groups, such as the trihydroxyflavone apigenin, the tetrahydroxyflavone kaempferol and the pentahydroxyflavone quercetin. In some embodiments, the substitution is at positions 6, 7, 8, 3', or 4', and combinations thereof. Preferred isoflavones comprise one or more hydroxyl and/or methoxy groups, such as the methoxy, dihydroxy isoflavone biochanin A.

The term "alkyl" as used herein refers to a straight, branched, or cyclic hydrocarbon chain fragment or radical containing between about one and about twenty carbon atoms, in another aspect between about one and about ten carbon atoms (e.g., methyl, ethyl, n-propyl, iso-propyl, cyclopropyl, n-butyl, iso-butyl, tert-butyl, cyclobutyl, adamantyl, noradamantyl and the like). Straight, branched, or cyclic hydrocarbon chains having eight or fewer carbon atoms will also be referred to herein as "loweralkyl". The hydrocarbon chains may further include one or more degrees of unsaturation, i.e., one or more double or triple bonds (e.g., vinyl, propargyl, allyl, 2-buten-1-yl, 2-cyclopenten-1-yl, 1,3-cyclohexadien-1-yl, 3-cyclohexen-1-yl and the like). Alkyl groups containing double bonds such as just described will also be referred to herein as "alkenes". Similarly, alkyl groups having triple bonds will also be referred to herein as "alkynes". However, as used in context with respect to cyclic alkyl groups, the combinations of double and/or triple bonds do not include those bonding arrangements that render the cyclic hydrocarbon chain aromatic.

In addition, the term "alkyl" as used herein further includes one or more substitutions at one or more carbon atoms of the hydrocarbon fragment or radical. Such substitutions include, but are not limited to: aryl; heterocycle; halogen (to form, e.g., trifluoromethyl, --CF_3); nitro (--NO_2); cyano(--CN); hydroxyl (also referred to herein as 'hydroxy"), alkoxyl (also referred herein as alkoxy) or aryloxyl (also referred to herein as "aryloxy", --OR); thio or mercapto, alkyl, or arylthio (--SR); amino, alkylamino, arylamino, dialkyl- or diarylamino, or arylalkylamino (--NRR'); aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, dialkylaminocarbonyl, diarylaminocarbonyl or arylalkylaminocarbonyl (--C(O)NRR'); carboxyl, or alkyl- or aryloxycarbonyl (--C(O)OR); carboxaldehyde, or aryl- or alkylcarbonyl (--C(O)R); iminyl, aryl- or alkyliminyl (--C(=NR)R'); sulfo (--SO_2OR); alkyl- or arylsulfonyl (--SO_2R); carbamido(--HNC(=O)NRR'); or thiocarbamido (--HNC(=S)NRR'); where R and R' independently are hydrogen, aryl or alkyl as defined herein. Substituents including heterocyclic groups (i.e., heterocycle, heteroaryl, and heteroaralkyl) are defined by analogy to the above-

described terms. For example, the term "heterocycloxy" refers to the group --OR, where R is heterocycle as defined below.

The term "alkoxyl" or "alkoxy" refers to the group --OR--, where R is substituted or unsubstituted alkyl, as defined above.

The term "methylene" refers to the group $-CH_2-$-.

The term "methine" refers to a methylene group for which one hydrogen atom has been replaced by a substituent as described above. The term "methine" can also refer to a methylene group for which one hydrogen atom is replaced by bond to form an sp^2 -hybridized carbon center (i.e., >C=O).

The term "halo" as used herein refers to the substituents fluoro, bromo, chloro, and iodo.

The term "carbonyl" as used herein refers to the functional group --C(O)--. However, it will be appreciated that this group may be replaced with well-known groups that have similar electronic and/or steric character, such as thiocarbonyl (--C(S)--); sulfinyl (--S(O)--); sulfonyl (--SO_2--), phosphonyl (--PO_2--), and methylene (-CH_2--). Other carbonyl equivalents will be familiar to those having skill in the medicinal and organic chemical arts. The term "carboxyl" refers to the group --C(O)OR, where R is hydrogen, an optionally substituted alkyl, as defined above, or a counterion.

The term "sulfo" as used herein refers to the substituent -$S(O)_2OR$, where R is hydrogen, an optionally substituted alkyl, as defined above, or a counterion.

II. Telomerase Inhibitors

As noted above, the immortalization of cells involves *inter alia* the activation of telomerase. More specifically, the connection between telomerase activity and the ability of many tumor cell lines, including skin, connective tissue, adipose, breast, lung, stomach, pancreas, ovary, cervix, uterus, kidney, bladder, colon, prostate, central nervous system (CNS), retina and blood tumor cell lines, to remain immortal has been demonstrated by analysis of

telomerase activity (Kim, et al., *supra*). This analysis, supplemented by data that indicates that the shortening of telomere length can provide the signal for replicative senescence in normal cells (see WO 93/23572), demonstrates that inhibition of telomerase activity can be an effective anti-cancer therapy. Thus, telomerase activity can prevent the onset of otherwise normal replicative senescence by preventing the normal reduction of telomere length and the concurrent cessation of cell replication that occurs in normal somatic cells after many cell divisions. In cancer cells, where the malignant phenotype is due to loss of cell cycle or growth controls or other genetic damage, an absence of telomerase activity permits the loss of telomeric DNA during cell division, resulting in chromosomal rearrangements and aberrations that lead ultimately to cell death. However, in cancer cells having telomerase activity, telomeric DNA is not lost during cell division, thereby allowing the cancer cells to become immortal, leading to a terminal prognosis for the patient. Agents capable of inhibiting telomerase activity in tumor cells offer therapeutic benefits with respect to a wide variety of cancers and other conditions (e.g., fungal infections) in which immortalized cells having telomerase activity are a factor in disease progression or in which inhibition of telomerase activity is desired for treatment purposes. The telomerase inhibitors of the invention can also be used to inhibit telomerase activity in germ line cells, which may be useful for contraceptive purposes.

Thus, in one aspect, the compounds of the present invention can provide a general method of treating many, if not most, malignancies, as demonstrated by the highly varied human tumor cell lines and tumors having telomerase activity. More importantly, the hydroxyflavone compounds of the present invention can be effective in providing treatments that discriminate between malignant and normal cells to a high degree, avoiding many of the deleterious side-effects present with most current chemotherapeutic regimes which rely on agents that kill dividing cells indiscriminately. The known 3-substituted flavone compounds and 2-substituted isoflavone compounds may generally be employed in this aspect of the present invention, including, for example, the hydroxyflavone derivatives 3,2'-

dihydroxyflavone (e.g., Indofine D-252, Indofine Chemical Company, Inc., Somerville, New Jersey U.S.A. ("Indofine")), 3,5,7-trihydroxyflavone (e.g., Indofine 02-1114S), 3,7,4'-trihydroxyflavone (e.g., Indofine T-251), 3,6,4'-trihydroxyflavone (e.g., Indofine T-405), 3,5,7,4',5'-pentahydroxy-flavone (e.g., Chembridge Corporation, San Diego, California U.S.A. ("Chembridge"), CHEMB22927), 3-hyroxyflavone (e.g., Aldrich Division, Sigma-Aldrich Co., Milwaukee, Wisconsin U.S.A. ("Aldrich"), 26,171-8), 7,3',4'-trihydroxyflavone (e.g., Indofine T-414), 3,5,7,3',4',5'-hexahydroxyflavone (e.g., Interbioscreen 1N-00524), 3,7,3',4'-tetrahydroxyflavone (e.g., Aldrich F50-5), 3,3'-dihydroxyflavone (e.g., Indofine D-602), 3,6-dihydroxyflavone (e.g., Aldrich 41,981-8), and 3,7-dihydroxyflavone (e.g., Indofine D-257).

III. Synthesis of Telomerase Inhibitors

The flavone compounds of the present invention can be synthesized using techniques and materials known to those of skill in the art, such as using the synthesis scheme represent in FIGURE 1 and described, for example, in Adam, W. et al, *J. Org. Chem.* **56**:7292 (1991), and Cushman, M. et al., *J. Med. Chem.* **37**:3353 (1994). The isoflavone compounds of the present invention can be synthesized using techniques and materials known to those of skill in the art, such as using the synthesis scheme represent in FIGURE 2 and described, for example, in Traxler, P. et al. *J. Med. Chem.* **42**:1018 (1999). Starting materials for the compounds of the invention may be obtained using standard techniques and commercially available precursor materials, such as those available from Aldrich Division, Sigma-Aldrich Co. (Milwaukee, Wis.), Sigma Division, Sigma-Aldrich Co. (St. Louis, Mo.), Lancaster Synthesis (Windham, N.H.), Apin Chemicals, Ltd. (New Brunswick, N.J.), Ryan Scientific (Columbia, S.C.), Maybridge (Cornwall, England), Arcos (Pittsburgh, Pa.), and Trans World Chemicals (Rockville, Md.).

The procedures described herein for synthesizing the compounds of the invention may include one or more steps of protection and deprotection (e.g., the formation and removal of acetal groups). In addition, the synthetic procedures disclosed below can include various

purifications, such as column chromatography, flash chromatography, thin-layer chromatography (TLC), recrystallization, distillation, high-pressure liquid chromatography (HPLC) and the like. Also, various techniques well known in the chemical arts for the identification and quantification of chemical reaction products, such as proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C NMR), infrared and ultraviolet spectroscopy (IR and UV), X-ray crystallography, elemental analysis (EA), HPLC and mass spectroscopy (MS) can be used as well. Methods of protection and deprotection, purification, identification and quantification are well known in the chemical arts.

IV. Anti-Tumor Activity of the Telomerase Inhibitors of the Invention

The compounds of the present invention demonstrate inhibitory activity against telomerase activity *in vitro*, as has been and can be demonstrated as described below. As used herein, the term "*in vitro*" refers to tests performed using living cells in tissue culture.

One method used to identify compounds of the invention that inhibit telomerase activity involves placing cells, tissues, or a cellular extract or other preparation containing telomerase in contact with several known concentrations of a test compound in a buffer compatible with telomerase activity. The level of telomerase activity for each concentration of test compound is measured and the IC_{50} (the concentration of the test compound at which the observed activity for a sample preparation was observed to fall one-half of its original or a control value) for the compound is determined using standard techniques. Other methods for determining the inhibitory concentration of a compound of the invention against telomerase can be employed as will be apparent to those of skill in the art based on the disclosure herein.

With the above-described methods, IC_{50} values for several of the compounds of the present invention were determined, and found to be below 100 μM.

With respect to the treatment of malignant diseases using the compounds described herein, compounds of the present invention are expected to induce crisis in telomerase-positive cell lines. Treatment of telomerase-positive cell lines, such as HEK-293 and HeLa cells, with a

compound of the invention is also expected to induce a reduction of telomere length in the treated cells.

Compounds of the invention are also expected to induce telomere reduction during cell division in human tumor cell lines, such as the ovarian tumor cell lines OVCAR-5 and SK-OV-3. Importantly, however, in normal human cells used as a control, such as BJ cells of fibroblast origin, the observed reduction in telomere length is expected to be no different from cells treated with a control substance, e.g., dimethyl sulfoxide (DMSO). The compounds of the invention also are expected to demonstrate no significant cytotoxic effects at concentrations below about 5 μM in the normal cells.

In addition, the specificity of the compounds of the present invention for telomerase can be determined by comparing their activity (IC_{50}) with respect to telomerase to other enzymes having similar nucleic acid binding or modifying activity similar to telomerase in vitro. Such enzymes include DNA Polymerase I, HeLa RNA Polymerase II, T3 RNA Polymerase, MMLV Reverse Transcriptase, Topoisomerase I, Topoisomerase II, Terminal Transferase and Single-Stranded DNA Binding Protein (SSB). Compounds having lower IC_{50} values for telomerase as compared to the IC_{50} values toward the other enzymes being screened are said to possess specificity for telomerase.

In vivo testing can also be performed using a mouse xenograft model, for example, in which OVCAR-5 tumor cells are grafted onto nude mice, in which mice treated with a compound of the invention are expected to have tumor masses that, on average, may increase for a period following the initial dosing, but will begin to shrink in mass with continuing treatment. In contrast, mice treated with a control (e.g., DMSO) are expected to have tumor masses that continue to increase.

From the foregoing those skilled in the art will appreciate that the present invention also provides methods for selecting treatment regimens involving administration of a compound of the invention. For such purposes, it may be helpful to perform a terminal restriction fragment (TRF) analysis in which DNA from tumor cells is analyzed by digestion with restriction

enzymes specific for sequences other than the telomeric $(T_2\ AG_3)_N$ sequence. Following digestion of the DNA, gel electrophoresis is performed to separate the restriction fragments according to size. The separated fragments are then probed with nucleic acid probes specific for telomeric sequences to determine the lengths of the terminal fragments containing the telomere DNA of the cells in the sample. By measuring the length of telomeric DNA, one can estimate how long a telomerase inhibitor should be administered and whether other methods of therapy (e.g., surgery, chemotherapy and/or radiation) should also be employed. In addition, during treatment, one can test cells to determine whether a decrease in telomere length over progressive cell divisions is occurring to demonstrate treatment efficacy.

V. Telomerase Inhibiting Compositions and Methods for Treating Diseases

The present invention also provides pharmaceutical compositions for treating cancer and other conditions in which inhibition of telomerase is an effective therapy. These compositions include a therapeutically effective amount of a telomerase inhibiting compound of the invention in a pharmaceutically acceptable carrier or salt.

In one preferred embodiment, the present invention provides compositions effective for treating cancer in a mammal. The compositions of the invention include a therapeutically effective amount of a compound of formulas I, II, III, IV or V (or a pharmaceutically acceptable salt thereof) in a pharmaceutically acceptable carrier. The compounds and compositions of the present invention may also be used for the treatment of other telomerase mediated conditions or diseases, such as, for example, other hyperproliferative or autoimmune disorders such as psoriasis, rheumatoid arthritis, immune system disorders requiring immune system suppression, immune system reactions to poison ivy or poison oak, and the like.

In addition, it will be appreciated that therapeutic benefits for treatment of cancer can be realized by combining a telomerase inhibitor of the invention with other anti-cancer agents, including other inhibitors of telomerase such as described in U.S. Patent Nos. 5,656,638, 5,760,062, 5,767,278, 5,770,613 and 5,863,936. The choice of such combinations will depend

on various factors including, but not limited to, the type of disease, the age and general health of the patient, the aggressiveness of disease progression, the TRF length and telomerase activity of the diseased cells to be treated and the ability of the patient to tolerate the agents that comprise the combination. For example, in cases where tumor progression has reached an advanced state, it may be advisable to combine a telomerase inhibiting compound of the invention with other agents and therapeutic regimens that are effective at reducing tumor size (e.g. radiation, surgery, chemotherapy and/or hormonal treatments). In addition, in some cases it may be advisable to combine a telomerase inhibiting agent of the invention with one or more agents that treat the side effects of a disease, e.g., an analgesic, or agents effective to stimulate the patient's own immune response (e.g., colony stimulating factor).

In one such method, a pharmaceutical formulation comprises a telomerase inhibitor of the invention with an anti-angiogenesis agent, such as fumagillin, fumagillin derivatives, or AGM-1470. The latter compound is available from Takeda Chemical Industries, Ltd., while the former compounds are described in Ingber, et al., 6 Dec. 1990, "Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth", Nature 348:555-557. Other combinations may include, but are not limited to, a telomerase inhibitor of the invention in addition to one or more antineoplastic agents or adjuncts (e.g., folinic acid or mesna).

Antineoplastic agents suitable for combination with the compounds of the present invention include, but are not limited to, alkylating agents including alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines, such as a benzodizepa, carboquone, meturedepa and uredepa; ethylenimines and methylmelamines such as altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolmelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cyclophosphamide, estramustine, iphosphamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichine, phenesterine, prednimustine, trofosfamide, and uracil mustard; nitroso ureas, such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine and ranimustine. Additional agents include dacarbazine, mannomustine, mitobronitol,

mitolactol and pipobroman. Still other classes of relevant agents include antibiotics, hormonal antineoplastics and antimetabolites. Yet other combinations will be apparent to those of skill in the art.

Additional agents suitable for combination with the compounds of the present invention include protein synthesis inhibitors such as abrin, aurintricarboxylic acid, chloramphenicol, colicin E3, cycloheximide, diphtheria toxin, edeine A, emetine, erythromycin, ethionine, fluoride, 5-fluorotryptophan, fusidic acid, guanylyl methylene diphosphonate and guanylyl imidodiphosphate, kanamycin, kasugamycin, kirromycin, and O-methyl threonine. Additional protein synthesis inhibitors include modeccin, neomycin, norvaline, pactamycin, paromomycine, puromycin, ricin, α-sarcin, shiga toxin, showdomycin, sparsomycin, spectinomycin, streptomycin, tetracycline, thiostrepton and trimethoprim. Inhibitors of DNA synthesis, including alkylating agents such as dimethyl sulfate, mitomycin C, nitrogen and sulfur mustards, MNNG and NMS; intercalating agents such as acridine dyes, actinomycins, adriamycin, anthracenes, benzopyrene, ethidium bromide, propidium diiodide-intertwining, and agents such as distamycin and netropsin, can also be combined with compounds of the present invention in pharmaceutical compositions. DNA base analogs such as acyclovir, adenine β-1-D-arabinoside, amethopterin, aminopterin, 2-aminopurine, aphidicolin, 8-azaguanine, azaserine, 6-azauracil, 2'-azido-2'-deoxynucleosides, 5-bromodeoxycytidine, cytosine β-1-D-arabinoside, diazooxynorleucine, dideoxynucleosides, 5-fluorodeoxycytidine, 5-fluorodeoxyuridine, 5-fluorouracil, hydroxyurea and 6-mercaptopurine also can be used in combination therapies with the compounds of the invention. Topoisomerase inhibitors, such as coumermycin, nalidixic acid, novobiocin and oxolinic acid, inhibitors of cell division, including colcemide, colchicine, vinblastine and vincristine; and RNA synthesis inhibitors including actinomycin D, α-amanitine and other fungal amatoxins, cordycepin (3'-deoxyadenosine), dichlororibofuranosyl benzimidazole, rifampicine, streptovaricin and streptolydigin also can be combined with the compounds of the invention to provide pharmaceutical compositions. In addition, tubulin assembly inhibitors such as paclitaxel (Taxol®) and related compounds that

promote the assembly of microtubules, resulting in highly stable, nonfunctional polymers, can be advantageously combined with the compounds of the invention to provide pharmaceutical compositions.

In another embodiment, the present invention includes compounds and compositions in which a telomerase inhibitor is either combined with or covalently bound to a cytotoxic agent bound to a targeting agent, such as a monoclonal antibody (e.g., a murine or humanized monoclonal antibody). It will be appreciated that the latter combination may allow the introduction of cytotoxic agents into cancer cells with greater specificity. Thus, the active form of the cytotoxic agent (i.e., the free form) will be present only in cells targeted by the antibody. Of course, the telomerase inhibitors of the invention may also be combined with monoclonal antibodies that have therapeutic activity against the targeted cells.

In addition to the application of the telomerase inhibitors of the present invention to the treatment of mammalian diseases characterized by telomerase activity, telomerase inhibitors such as those disclosed herein, can be applied to agricultural phytopathogenic organisms that are characterized by telomerase activity. These organisms include nematodes such as *Ceanorhabditis elegans*, in which telomerase activity has been found, and in fungi which are expected to have telomerase activity based on the determination that the DNA of the fungus *Ustilago maydis* exhibits telomeres having the tandem TTAGGG repeats that are maintained by telomerase. Also, protozoans have TTAGGG telomeres and cause human disease. The telomerase-inhibiting compounds of the invention can be administered to plants and soil infected with phytopathogenic organisms having telomerase activity alone, or in combination with other telomerase-inhibiting agents and/or other agents used to control plant diseases. The determination of the compositions used to control such phytopathogenic organisms and the appropriate modes of delivering such compositions will be known to those having skill in the agricultural arts.

The determination that nematodes, protozoans and possibly fungi have telomerase activity also indicates that the telomerase inhibitors provided by the present invention can be

used to treat nematode infections in humans and animals of veterinary interest such as dogs and cats. Nematode infection in humans and animals often is in the form of hookworm or roundworm infection and leads to a host of deadly secondary illnesses such as meningitis, myocarditis, and various neurological diseases. Thus, it will be appreciated that administration of the telomerase-inhibiting compounds such as those of the invention, alone, or in combination with other telomerase-inhibiting agents and/or other therapeutic agents, can be used to control nematode, protozoan and fungal infections in humans and animals.

In general, a suitable effective dose of a compound of the invention will be in the range of 0.001 to 1000 milligram (mg) per kilogram (kg) of body weight of the recipient per day, in another aspect in the range of 0.001 to 100 mg per kg of body weight per day, in yet another aspect between about 0.1 and 100 mg per kg of body weight per day and in yet another aspect in the range of between 0.1 to 10 mg per kg of body weight per day. The desired dosage may be presented in one, two, three, four, or more subdoses administered at appropriate intervals throughout the day, or by the action of a continuous or on-demand pump delivery system. These subdoses can be administered as unit dosage form, for example, containing from 5 to 10,000 mg, or in another aspect from 10 to 1000 mg of active ingredient per unit dosage from.

The composition used in these therapies can be in a variety of forms. These include, for example, solid, semi-solid, and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspensions, liposomes, and injectable and infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application. The compositions may also include conventional pharmaceutically acceptable carriers and adjuvants, as is well known to those of skill in the art. See, e.g., REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Co.: Easton, Pa., 17th Ed. (1985). In one aspect of the invention, administration will be by oral or parenteral (including subcutaneous, intramuscular, intravenous, and intradermal) routes. The therapeutic methods and agents of this invention can of course be used concomitantly or in combination with other methods and agents for treating a particular disease or disease condition.

While it is possible to administer the active ingredient of this invention alone, it is preferable to present a therapeutic agent as part of a pharmaceutical formulation or composition. The formulations of the present invention comprise at least one telomerase activity-inhibiting compound of this invention in a therapeutically or pharmaceutically effective dose together with one or more pharmaceutically or therapeutically acceptable carriers and optionally other therapeutic ingredients. Various considerations for preparing such formulations are described, e.g., in Gilman et al. (eds.) GOODMAN AND GILMAN'S: THE PHARMACOLOGICAL BASES OF THERAPEUTICS, 8th Ed., Pergamon Press (1 990); and REMINGTON'S, *supra*. Methods for administration are discussed therein, e.g., for oral, intravenous, intraperitoneal, intramuscular, and other forms of administration. Typically, methods for administering pharmaceutical compositions will be either topical, parenteral, or oral administration methods for prophylactic and/or therapeutic treatment. Oral administration is preferred. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. As noted above, unit dosage forms suitable for oral administration include powders, tablets, pills, and capsules.

Where appropriate, topical administration may be used to deliver a compound of the invention, for example by percutaneous passage of the drug into the systemic circulation of the patient. The skin sites include anatomic regions for transdermally administering the drug, such as the forearm, abdomen, chest, back, buttock, and mastoidal area. The compound is administered to the skin by placing on the skin either a topical formulation comprising the compound or a transdermal drug delivery device that administers the compound. In either embodiment, the delivery vehicle is designed, shaped, sized, and adapted for easy placement and comfortable retention on the skin.

A variety of transdermal drug delivery devices can be employed with the compounds of this invention. For example, a simple adhesive patch comprising a backing material and an acrylate adhesive can be prepared. The drug and any penetration enhancer can be formulated into the adhesive casting solution. The adhesive casting solution can be cast directly onto the

backing material or can be applied to the skin to form an adherent coating. See, e.g., U.S. Pat. Nos. 4,310,509; 4,560,555; and 4,542,012.

In other embodiments, the compound of the invention will be delivered using a liquid reservoir system drug delivery device. These systems typically comprise a backing material, a membrane, an acrylate based adhesive, and a release liner. The membrane is sealed to the backing to form a reservoir. The drug or compound and any vehicles, enhancers, stabilizers, gelling agents, and the like are then incorporated into the reservoir. See, e.g., U.S. Pat. Nos. 4,597,961; 4,485,097; 4,608,249; 4,505,891; 3,843,480; 3,948,254; 3,948,262; 3,053,255; and 3,993,073.

Matrix patches comprising a backing, a drug/penetration enhancer matrix, a membrane, and an adhesive can also be employed to deliver a compound of the invention transdermally. The matrix material typically will comprise a polyurethane foam. The drug, any enhancers, vehicles, stabilizers, and the like are combined with the foam precursors. The foam is allowed to cure to produce a tacky, elastomeric matrix which can be directly affixed to the backing material. See, e.g., U.S. Pat. Nos. 4,542,013; 4,460,562; 4,466,953; 4,482,534; and 4,533,540.

Also included within the invention are preparations for topical application to the skin comprising a compound of the invention, typically in concentrations in the range from about 0.001% to 10%, together with a non-toxic, pharmaceutically acceptable topical carrier. These topical preparations can be prepared by combining an active ingredient according to this invention with conventional pharmaceutical diluents and carriers commonly used in topical dry, liquid, and cream formulations. Ointment and creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Such bases may include water and/or an oil, such as liquid paraffin or a vegetable oil, such as peanut oil or castor oil. Thickening agents that may be used according to the nature of the base include soft paraffin, aluminum stearate, cetostearyl alcohol, propylene glycol, polyethylene glycols, woolfat, hydrogenated lanolin, beeswax, and the like.

Lotions may be formulated with an aqueous or oily base and will, in general, also include one or more of the following: stabilizing agents, emulsifying agents, dispersing agents, suspending agents, thickening agents, coloring agents, perfumes, and the like. Powders may be formed with the aid of any suitable powder base, e.g., talc, lactose, starch, and the like. Drops may be formulated with an aqueous base or non-aqueous base also comprising one or more dispersing agents, suspending agents, solubilizing agents, and the like. Topical administration of compounds of the invention may also be preferred for treating diseases such as skin cancer and fungal infections of the skin (pathogenic fungi typically express telomerase activity).

The topical pharmaceutical compositions according to this invention may also include one or more preservatives or bacteriostatic agents, e.g., methyl hydroxybenzoate, propyl hydroxybenzoate, chlorocreosol, benzalkonium chlorides, and the like. The topical pharmaceutical compositions also can contain other active ingredients such as antimicrobial agents, particularly antibiotics, anesthetics, analgesics, and antipruritic agents.

The compounds of the present invention can also be delivered through mucosal membranes. Transmucosal (i.e., sublingual, buccal, and vaginal) drug delivery provides for an efficient entry of active substances to systemic circulation and reduces immediate metabolism by the liver and intestinal wall flora. Transmucosal drug dosage forms (e.g., tablet, suppository, ointment, pessary, membrane, and powder) are typically held in contact with the mucosal membrane and disintegrate and/or dissolve rapidly to allow immediate systemic absorption. Note that certain such routes may be used even where the patient is unable to ingest a treatment composition orally. Note also that where delivery of a telomerase inhibitor of the invention would be enhanced, one can select a composition for delivery to a mucosal membrane, e.g., in cases of colon cancer one can use a suppository to deliver the telomerase inhibitor.

For delivery to the buccal or sublingual membranes, typically an oral formulation, such as a lozenge, tablet, or capsule, will be used. The method of manufacture of these formulations is known in the art, including, but not limited to, the addition of the pharmacological agent to a

pre-manufactured tablet; cold compression of an inert filler, a binder, and either a pharmacological agent or a substance containing the agent (as described in U.S. Pat. No. 4,806,356); and encapsulation. Another oral formulation is one that can be applied with an adhesive, such as the cellulose derivative hydroxypropyl cellulose, to the oral mucosa, for example as described in U.S. Pat. No. 4,940,587. This buccal adhesive formulation, when applied to the buccal mucosa, allows for controlled release of the pharmacological agent into the mouth and through the buccal mucosa.

Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly, or intravenously. Thus, this invention provides compositions for intravenous administration that comprise a solution of a compound of the invention dissolved or suspended in an acceptable carrier. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, buffered water, saline, dextrose, glycerol, ethanol, or the like. These compositions will be sterilized by conventional, well known sterilization techniques, such as sterile filtration. The resulting solutions can be packaged for use as is or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, etc. Such formulations will be useful in treating ovarian cancers.

Another method of parenteral administration employs the implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained. See, e.g., U.S. Pat. No. 3,710,795.

Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc., an active compound as defined above and optional pharmaceutical adjuvants in an excipient, such as, for example, water, saline, aqueous dextrose, glycerol,

ethanol, olive oil, and other lipophilic solvents, and the like, to form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known and will be apparent to those skilled in this art; for example, see REMINGTON'S PHARMACEUTICAL SCIENCES, *supra*. The composition or formulation to be administered will contain an effective amount of an active compound of the invention.

For solid compositions, conventional nontoxic solid carriers can be used and include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 0.1-95% of active ingredient, in one aspect of the invention about 20% of active ingredient.

The compositions containing the compounds of the invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, as described above, in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective amount or dose." Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient.

In addition to internal (*in vivo*) administration, the compounds and compositions of the invention may be applied *ex vivo* to achieve therapeutic effects, as for example, in the case of a patient suffering from leukemia. In such an application, cells to be treated, e.g., blood or bone marrow cells, are removed from a patient and treated with a pharmaceutically effective amount of a compound of the invention. The cells are returned to the patient following treatment. Such

a procedure can allow for exposure of cells to concentrations of therapeutic agent for longer periods or at higher concentrations than otherwise available.

Once improvement of the patient's conditions has occurred, as, for example, by the occurrence of remission in the case of a cancer patient, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, can be reduced, as a function of the systems, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment can cease. Patients can, however, require additional treatment upon any recurrence of the disease symptoms.

In prophylactic applications (e.g. chemoprevention), compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective amount or dose." In this use, the precise amounts again depend on the patient's state of health and weight.

As will be apparent to those of skill in the art upon reading of this disclosure, the present invention provides valuable reagents relating to human and mammalian telomerase. The above description of necessity provides a limited and merely illustrative sampling of specific compounds, and should not be construed as limiting the scope of the invention. Other features and advantages of the invention will be apparent from the following examples and claims.

EXAMPLES

The following examples describe specific aspects of the invention to illustrate the invention and also provide a description of methods that can be used to identify and test compounds that inhibit the activity of telomerase to aid those of skill in the art in understanding and practicing the invention. The examples should not be construed as limiting the invention in any manner.

Example 1

Commercially Available Compounds of the Invention

The following representative compounds useful in the practice of the invention are commercially available from the indicated sources:

Compound Name	Commercial Source
3,2'-dihydroxyflavone	Indofine D-252
3,5,7-trihydroxyflavone	Indofine 02-1114S
3,7,4'-trihydroxyflavone	Indofine T-251
3,6,4'-trihydroxyflavone	Indofine T-405
3,5,7,4',5'-pentahydroxy-flavone	Chembridge[2] CHEMB22927
3-hyroxyflavone	Aldrich[3] 26,171-8
3,5,7,3',4',5'-hexahydroxyflavone	Interbioscreen 1N-00524
3,7,3',4'-tetrahydroxyflavone	Aldrich F50-5
3,3'-dihydroxyflavone	Indofine D-602
3,6-dihydroxyflavone	Aldrich 41,981-8
3,7-dihydroxyflavone	Indofine D-257

Example 2

Preparation of Affinity Purified Extract

Extracts used for screening telomerase inhibitors were routinely prepared from 293 cells over-expressing the protein catalytic subunit of telomerase (hTERT; see WO 98/14593). These cells were found to have 2-5 fold more telomerase activity than parental 293 cells. 200 ml of packed cells (harvested from about 100 liters of culture) were resuspended in an equal volume of hypotonic buffer (10 mM Hepes pH 7.9, 1 mM $MgCl_2$, 1 mM DTT, 20 mM KCl, 1 mM

PMSF) and lysed using a dounce homogenizer. The glycerol concentration was adjusted to 10% and NaCl was slowly added to give a final concentration of 0.3 M. The lysed cells were stirred for 30 min and then pelleted at 100,000 x g for 1 hr. Solid ammonium sulfate was added to the S100 supernatant to reach 42% saturation. The material was centrifuged; the pellet was resuspended in one fifth of the original volume and dialyzed against Buffer 'A' containing 50 mM NaCl. After dialysis the extract was centrifuged for 30 min at 25,000 x g. Prior to affinity chromatography, Triton X-100 (0.5 %), KCl (0.3 M) and tRNA (50 µg/ml) were added. Affinity oligo (5' biotinTEG-biotinTEG-biotinTEG-GTA GAC CTG TTA CCA guu agg guu ag 3'; lower case represents 2' O-methyl ribonucleotides and upper case represents deoxynucleotides) was added to the extract (1 nmol per 10 ml of extract). After an incubation of 10 min at 30 °C, Neutravidin beads (Pierce; 250 µl of a 50% suspension) were added and the mixture was rotated overnight at 4 °C. The beads were pelleted and washed three times with Buffer 'B' containing 0.3 M KCl, twice with Buffer 'B' containing 0.6 M KCl, and twice more with Buffer B containing 0.3 M KCl. Telomerase was eluted in Buffer 'B' containing 0.3 M KCl, 0.15% Triton X-100 and a 2.5 molar excess of displacement oligo (5'-CTA ACC CTA ACT GGT AAC AGG TCT AC-3' at 0.5 ml per 125 µl of packed Neutravidin beads) for 30 min. at room temperature. A second elution was performed and pooled with the first. Purified extracts typically had specific activities of 10 fmol nucleotides incorporated/min/µl extract, or 200 nucleotides/min/mg total protein.

Buffer 'A'	**Buffer 'B'**
20 mM Hepes pH 7.9	20 mM Hepes pH 7.9
1 mM $MgCl_2$	1 mM EDTA
1 mM DTT	1 mM DTT
1 mM EGTA	10% glycerol
10 % glycerol	0.5 Triton X-100

Example 3

Telomerase Specific Activity Determination

Three separate 100 µl telomerase assays are set up with the following buffer solutions: 50 mM Tris acetate, pH 8.2, 1 mM DTT, 1 mM EGTA, 1 mM $MgCl_2$, 100 mM K acetate, 500 µM dATP, 500 µM TTP, 10 µM ^{32}P-dGTP (25 Ci/mmol), and 100 nM $d(TTAGGG)_3$. To the individual reactions 2.5, 5 or 10 µl of affinity-purified telomerase (see Example 2) is added and the reactions are incubated at 37 °C. At 45 and 90 minutes, 40 µl aliquots are removed from each reaction and added to 160 µl of Stop Buffer (100mM NaCl, 10 mM Na pyrophosphate, 0.2% SDS, 2 mM EDTA, 100 µg/ml tRNA). 10 µl trichloroacetic acid (TCA) (100%) is added and the sample is incubated on ice for 30 minutes. The sample is pelleted in a microcentrifuge (12000 x g force) for 15 minutes. The pellet is washed with 1 ml 95% ethanol and pelleted again in the microcentrifuge (12000 x g force) for 5 minutes. The pellet is resuspended in 50 µl dH_20 and transferred to a 12 x 75 glass test tube containing 2.5 ml of ice cold solution of 5% TCA and 10 mM Na pyrophosphate. The sample is incubated on ice for 30 minutes. The sample is filtered through a 2.5 cm wet (dH_2O) GFC membrane (S&S) on a vacuum filtration manifold. The filter is washed three times under vacuum with 5 ml ice cold 1% TCA, and once with 5 ml 95% ethanol. The filter is dried and counted in a scintillation counter using scintillation fluid. The fmol of nucleotide incorporated is determined from the specific activity of radioactive tracer. The activity of extract is calculated based on the dNTP incorporated and is expressed as fmol dNTP/min/µl extract.

Example 4

Telomerase Activity Assay

Bio-Tel FlashPlate Assay

An assay is provided for the detection and/or measurement of telomerase activity by measuring the addition of TTAGGG telomeric repeats to a biotinylated telomerase substrate primer; a reaction catalyzed by telomerase. The biotinylated products are captured in

streptavidin coated microtiter plates. An oligonucleotide probe complementary to 3.5 telomere repeats labeled with [^{33}P] is used for measuring telomerase products, as described below. Unbound probe is removed by washing and the amount of probe annealing to the captured telomerase products is determined by scintillation counting.

Method:

1. Compounds are stored as concentrated stocks and dissolved in 100 % dimethylsulfoxide (DMSO).
2. For testing, the compounds are diluted to a 15X working stock in 50% DMSO and 2 µl is dispensed into two wells of a 96-well microtiter dish (assayed in duplicate).
3. Telomerase extract is diluted to a specific activity of 0.04 - 0.09 fmol dNTP incorporated/min./µl in Telomerase Dilution Buffer and 18 µl added to each sample well to preincubate with compound for 30 minutes at room temperature.
4. The telomerase reaction is initiated by addition of 10 µl Master Mix to the wells containing telomerase extract and compound. The plates are sealed and incubated at 37°C for 90 min.
5. The reaction is stopped by the addition of 10 µl HCS.
6. 25 µl of the reaction mixture is transferred to a 96-well streptavidin-coated FlashPlate (NEN) and incubated for 2 hours at room temperature with mild agitation.
7. The wells are washed three times with 180 µl 2X SSC without any incubation.
8. The counts of probe annealed to biotinylated telomerase products are detected on a scintillation counter.

Buffers:

Telomerase Dilution Buffer

50 mM Tris-acetate, pH 8.2
1 mM DTT
1 mM EGTA
1 mM $MgCl_2$
830 nM BSA

Master Mix (MM)

50 mM Tris-acetate, pH 8.2

1 mM DTT

1 mM EGTA

1 mM $MgCl_2$

150 mM K acetate

10 μM dATP

20 μM dGTP

120 μM dTTP

100 nM biotinylated primer (5'-biotin-AATCCGTCGAGCAGAGTT-3')

5.4 nM labeled probe [5'-CCCTAACCCTAACCCTAACCC-(^{33}P) A_{1-50} - 3']; specific activity approximately 10^9 cpm/μg or higher

Hybridization Capture Solution (HCS)

12X SSC (1X = 150 mM NaCl/30 mM Na_3Citrate)

40 mM EDTA

40 mM Tris-HCl, pH 7.0

Using the foregoing assay, the compounds of Example 1 were shown to have telomerase IC_{50} values below 100 μM.

Example 5

Anti-tumor Activity

Reduction of Telomere Length in Tumor Cells

Colonies of the tumor cell lines, such as the ovarian tumor cell lines OVCAR-5 and SK-OV-3, and normal human cells used as a control (e.g., normal human BJ cells) are prepared using standard methods and materials. In one test, the colonies are prepared by seeding 15-centimeter dishes with about 10^6 cells in each dish. The dishes are incubated to allow the cell colonies to grow to about 80% confluence, at which time each of the colonies are divided into

two groups. One group is exposed to a subacute dose of a compound of the invention at a predetermined concentration (e.g., between about 5 μM and about 20 μM) for a period of about 4-8 hours after plating following the split; the other group is exposed to a control (e.g., DMSO).

Each group is then allowed to continue to divide, and the groups are split evenly again (near confluence). The same number of cells are seeded for continued growth. The compound or control is added every fourth day to the samples at the same concentration delivered initially. Remaining cells are analyzed for telomere length. As the untested cell cultures near confluence, the samples are split again as just described. This sequence of cell doubling and splitting is continued for about 20 to 25 doublings. Thus, a determination of telomere length as a function of cell doublings is obtained.

Telomere length is determined by digesting the DNA of the cells using restriction enzymes specific for sequences other than the repetitive T_2AG_3 sequence of human telomeres (TRF analysis). The digested DNA is separated by size using standard techniques of gel electrophoresis to determine the lengths of the telomeric repeats, which appear, after probing with a telomere DNA probe, on the gel as a smear of high-molecular weight DNA (approximately 2 Kb-15 Kb).

The results of the telomere length analysis are expected to indicate that the compounds of the invention have no affect on the rate of decrease in telomere length for control cells as a function of progressive cell doublings. With respect to the tumor cell lines, however, measurable decreases in telomere length are expected to be determined for tumor cells exposed to the compounds of the invention. Tumor cells exposed to the control are expected to maintain steady telomere lengths. Thus, the compounds of the invention are expected to cause resumption of the normal loss of telomere length as a function of cell division in tumor cells.

In another experiment, HEK-293 cells are incubated with a compound of the invention and a control at concentrations between about 1 μM and about 20 μM using the protocol just described. Cells are expected to enter crisis (i.e., the cessation of cell function) within several weeks following administration of the test compound of the invention. In addition, TRF

analysis of the cells using standard methodology is expected to show that the test compounds of the invention are effective in reducing telomere length. In addition to the HEK-293 cells described above, this assay can be performed with any telomerase-positive cell line, such as HeLa cells.

Specificity

Compounds of the invention are screened for activity (IC_{50}) against telomerase and several enzymes having nucleic acid binding or modifying activities related to telomerase using standard techniques. The enzymes being screened include Telomerase, DNA Polymerase I, HeLa RNA Polymerase II, T3 RNA Polymerase, MMLV Reverse Transcriptase, Topoisomerase I, Topoisomerase II, Terminal Transferase and Single-Stranded DNA Binding Protein (SSB). The specificity of a compound of the invention for telomerase is determined by comparing the IC_{50} of the compound with respect to telomerase with the IC_{50} values of the compound for each of the enzymes being screened. The compound is determined to have high specificity for telomerase if the IC_{50} for telomerase of the compound is lower than the IC_{50} values for each of the enzymes being screened.

Cytotoxicity

The XTT assay for cytotoxicity (see Weislow et al., *J. National Cancer Inst.* 81: 577-586, (1989); Scudiero et al, "Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines", *Cancer Res.* 48:4827-4833, 1988) is performed using HeLa cells. The cell lines used in the assay are exposed to a compound of the invention for 72 hours at concentrations ranging from about 1 μM to about 1,000 μM. During this period, the optical density (OD) of the samples is determined for light at 540 nanometers (nm). No significant cytotoxic effects are expected to be observed at concentrations less than about 5 μM. It will be appreciated that other tumor cells lines such as the ovarian tumor cell lines OVCAR-5 and SK-OV-3 can be used to determine cytotoxicity in addition to control cell lines such as normal human BJ cells. Other assays for cytotoxicity such

as the MTT assay (see Berridge et al., 1996, *Biochemica* 4:14-19) and the alamarBlue™ assay (U.S. Patent No. 5,501,959) can be used as well.

Some compounds may induce G2 arrest at concentrations above about 5 μM (i.e., at 10 μM-20 μM concentrations or higher). To observe any telomerase inhibiting effects the compounds should be administered at a concentration below the level of cytotoxicity. Nevertheless, since the effectiveness of many cancer chemotherapeutics derives from their cytotoxic effects, it is within the scope of the present invention that the compounds of the present invention be administered at any dose for which chemotherapeutic effects are observed.

Anti-tumor Activity

A human tumor xenograft model in which OVCAR-5 tumor cells are grafted into nude mice can be constructed using standard techniques and materials. The mice are divided into two groups. One group is treated intraperitoneally with a compound of the invention. The other group is treated with a control comprising a mixture of either DMSO or ethanol and emulphor (oil) and phosphate buffer solution (PBS). The average tumor mass for mice in each group is determined periodically following the xenograft using standard methods and materials.

In the group treated with a compound of the invention, the average tumor mass is expected to increase following the initial treatment for a period of time, after which time the tumor mass is expected to stabilize and then begin to decline. Tumor masses in the control group are expected to increase throughout the study. Thus, the compounds of the invention are expected to lessen dramatically the rate of tumor growth and ultimately induce reduction in tumor size and elimination of the tumor.

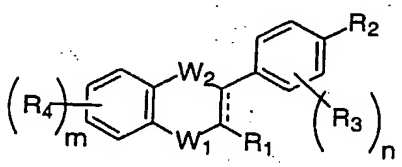
Thus, the present invention provides novel compounds, compositions and methods for inhibiting telomerase activity and treating disease states in which telomerase activity has deleterious effects, especially cancer. The compounds of the invention provide a highly selective and effective treatment for malignant cells that require telomerase activity to remain immortal; yet, without affecting non-malignant cells.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

We claim:

1. A method of inhibiting telomerase activity in a cell, comprising contacting the cell with an effective amount of a telomerase inhibiting substituted flavone or isoflavone compound.

2. The method of Claim 1 wherein the telomerase inhibiting substituted flavone compound is a compound of the formula (I):



(I)

wherein one of W_1 and W_2 is C=O and the other of W_1 and W_2 is O or N;

⋰ is a single or double bond;

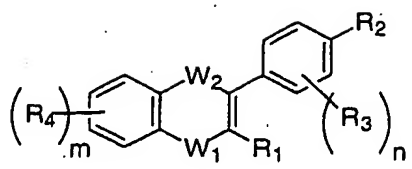
R_1 is hydroxyl, carboxyl or sulfo;

R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen, halo, alkyl, haloalkyl, hydroxyl, alkoxyl, cyano, nitro, amino, alkylamino, dialkylamino, carboxyl, and alkoxycarbonyl;

m and n are independently 0, 1, 2, 3 or 4;

and the pharmaceutically acceptable salts thereof.

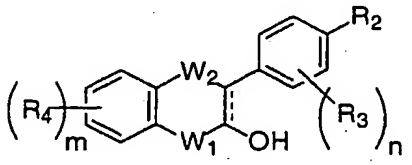
3. The method of Claim 2 wherein the telomerase inhibiting substituted flavone compound is a compound of the formula:



(II)

or a pharmaceutically acceptable salt thereof.

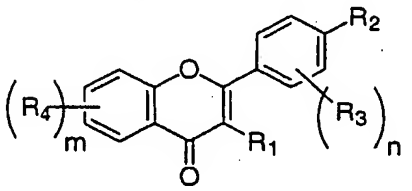
4. The method of Claim 2 wherein the telomerase inhibiting substituted flavone compound is a compound of the formula:



(III)

or a pharmaceutically acceptable salt thereof.

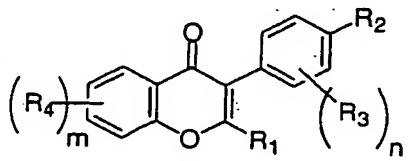
5. The method of Claim 2 wherein the telomerase inhibiting substituted flavone compound is a compound of the formula:



(IV)

or a pharmaceutically acceptable salt thereof.

6. The method of Claim 2 wherein the telomerase inhibiting substituted flavone compound is a compound of the formula:

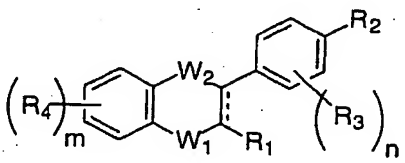


(V)

or a pharmaceutically acceptable salt thereof.

7. The method of Claim 2 wherein the telomerase inhibiting substituted flavone compound is selected from the group consisting of 3,2'-dihydroxyflavone, 7,4'-dihydroxyflavone, 3,5,7-trihydroxyflavone, 3,7,4'-trihydroxyflavone, 3,6,4'-trihydroxyflavone, 3,5,7,4',5'-pentahydroxy-flavone, 3-hyroxyflavone, 3,5,7,3',4',5'-hexahydroxyflavone, 3,7,3',4'-tetrahydroxyflavone, 3,3'-dihydroxyflavone, 3,6-dihydroxyflavone and 3,7-dihydroxyflavone.

8. A composition comprising an amount effective to inhibit telomerase activity in a cell of a telomerase inhibiting substituted flavone compound having a structure of formula (I):



(I)

wherein one of W_1 and W_2 is C=O and the other of W_1 and W_2 is O or N;

⋰ is a single or double bond;

R_1 is hydroxyl, carboxyl or sulfo;

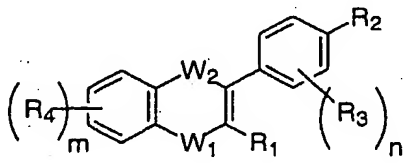
R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen, halo, alkyl, haloalkyl, hydroxyl, alkoxyl, cyano, nitro, amino, alkylamino, dialkylamino, carboxyl, and alkoxycarbonyl;

m and n are independently 0, 1, 2, 3 or 4;

and the pharmaceutically acceptable salts thereof;

together with a pharmaceutically acceptable carrier.

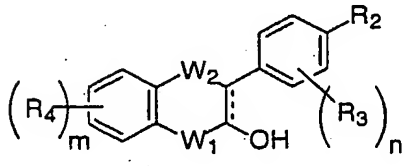
9. A composition of Claim 8 wherein the compound has the formula:



(II)

or a pharmaceutically acceptable salt thereof.

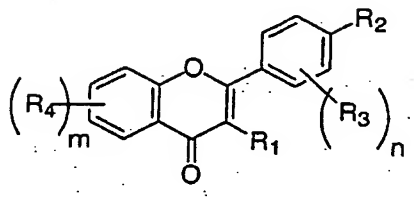
10. A composition of Claim 8 wherein the compound has the formula:



(III)

or a pharmaceutically acceptable salt thereof.

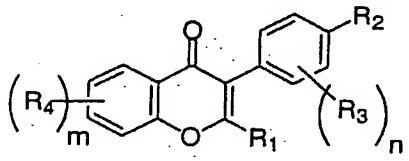
11. A composition of Claim 8 wherein the compound has the formula:



(IV)

or a pharmaceutically acceptable salt thereof.

12. A composition of Claim 8 wherein the compound has the formula:



(V)

or a pharmaceutically acceptable salt thereof.

13. A composition of Claim 8 wherein the compound is selected from the group consisting of 3,2'-dihydroxyflavone, 7,4'-dihydroxyflavone, 3,5,7-trihydroxyflavone, 3,7,4'-trihydroxyflavone, 3,6,4'-trihydroxyflavone, 3,5,7,4',5'-pentahydroxy-flavone, 3-hyroxyflavone, 3,5,7,3',4',5'-hexahydroxyflavone, 3,7,3',4'-tetrahydroxyflavone, 3,3'-dihydroxyflavone, 3,6-dihydroxyflavone and 3,7-dihydroxyflavone.

14. A composition according to any one of claims 8 to 13 for use in inhibition of telomerase activity.

15. A composition according to any one of claims 8 to 13 for use in inhibition of proliferation of a telomerase positive cell.

16. The use of a composition according to any one of claims 8 to 13 in the manufacture of a medicament for inhibition of telomerase activity.

17. The use of a composition according to any one of claims 8 to 13 in the manufacture of a medicament for inhibition of telomerase activity in a cell.

18. The use of a composition according to any one of claims 8 to 13 in the manufacture of a medicament for the treatment of a telomerase mediated condition or disease.

19. A composition according to any one of claims 8 to 13 for use in treating a tumour.

20. The use of a composition according to any one of claims 8 to 13 in the manufacture of a medicament for the treatment of a tumour.

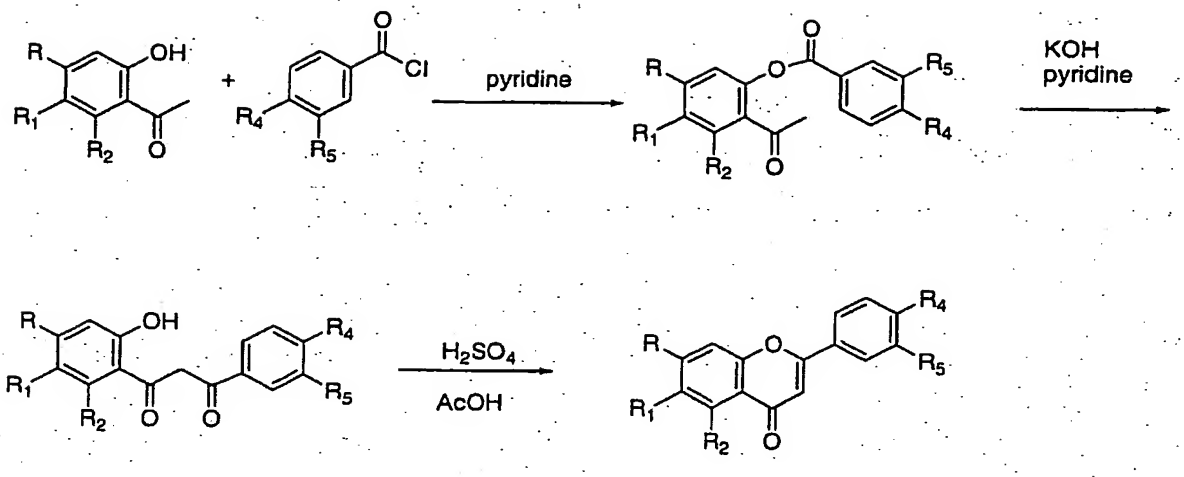


FIGURE 1.

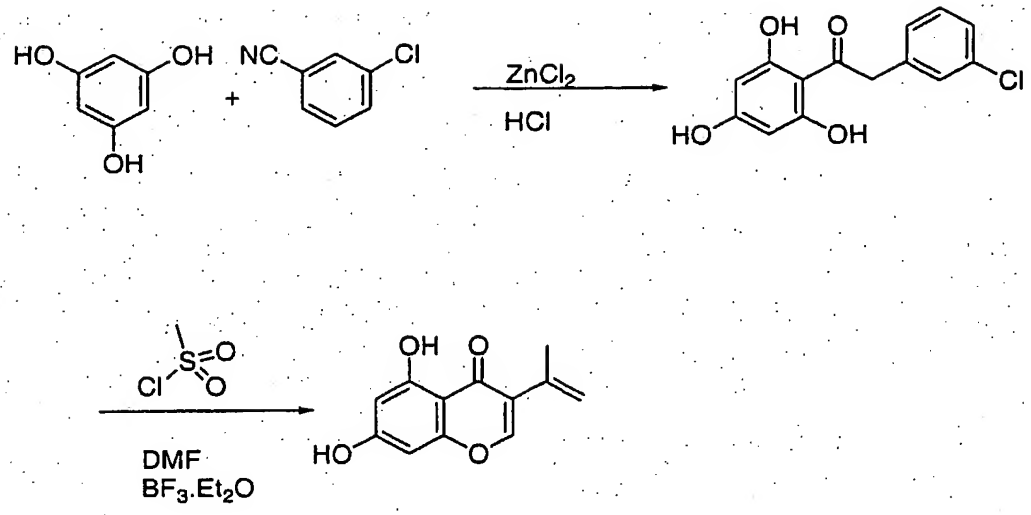


FIGURE 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/13419

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 31/35, 31/47
US CL : 514/312, 456
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/312, 456

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
REGISTRY, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database CAPLUS on STN, (Columbus, OH, USA), No. 123:65858, KREUTZ, W. ET AL. 'Alkalinizing infusions as immunostimulants for treatment of cancer,' abstract, DE 4407484 A1, 08 May 1995.	1-20
X	Database CAPLUS on STN, (Columbus, OH, USA), No. 117:55958, MIYOSHI, T. 'Antitumor composition containng a kaempferol-type aromatic hydrocarbon, catalase, and iron,' abstract, JP 04103529 A2, 06 April 1992.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
06 JUNE 2001

Date of mailing of the international search report
22 JUN 2001

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